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## Pulsed electrochemical technique for monitoring antibody–antigen reactions at interfaces

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### Abstract

The mechanism of pulsed potential waveform for monitoring antibody–antigen interactions at immunosensor interfaces is discussed. Some examples of antibody–antigen interactions at quartz crystal microbalance and polymer-modified antibody electrodes are presented. The binding and release of analytes to the modified sensor surfaces are monitored in real time. An overview of the analytical performance of some recently developed sensors utilizing pulsed amperometric detection techniques is also presented. The sensors obtained are reversible and reproducible (with a relative standard deviation of 0.30%), and their response times are in the millisecond range. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Immunosensors; Antibody–antigen interactions; Pulsed amperometric detection; Reversibility

### 1. Introduction

The specificity of antibody–antigen (Ab–Ag) interactions as well as the possibility of developing antibodies for a variety of small molecules or synthetic fragments of complex biological antigens have opened the way for the development of immunosensor devices for clinical, environmental, military, and food analysis applications [1–3]. Radioisotope labels have been used in clinical analysis due to their low sensitivities and the availability of automated equipment. However, the inconvenience of handling or disposing radioactive residues is drawing attention to other methods of labeling. Notable among these are the enzyme-linked immunosorbent assays (ELISA) [4],

fluorescence [5], chemiluminescence [6], electrochemical potential measurements [7], piezoelectric [8] and surface plasmon resonance (SPR) techniques [9]. All of these represent the physical means of transduction of Ab–Ag complexes.

The electrochemical detection of Ab–Ag interactions continues to be the subject of several research efforts. Capacitance changes at electrode–electrolyte interfaces resulting from Ab–Ag recognition have been used to develop capacitive immunosensors [10]. Also, the voltammetric [11], amperometric [12] and potentiometric [13] detection of Ab–Ag interactions have been accomplished through the application of redox-modified antigens or antibodies to electrode interfaces in the presence of analyte substances.

The major obstacles in adapting immunochemistry to immunosensor development, especially using

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“label-free” techniques are (i) immobilization procedures that usually lead to a partial loss of antibody binding capacity, and (ii) regeneration (i.e., release of the Ab–Ag binding) that is most often incomplete or impossible due to the lability of the Ab. However, regeneration is necessary in order to be able to calibrate the sensor. Therefore, several attempts have been made to facilitate the release of Ab–Ag binding or to regenerate Ab-coated surfaces. These include lowering the pH, increasing the ionic strength of the solution, or simply replacing the previously bound Ag with another compound of higher affinity [2,14].

Recently we have addressed the use of conducting polymer layers assembled on solid electrodes for the amperometric detection of various compounds [3,15,16]. We demonstrated the general method of immobilizing Ab or Ag on conducting polymer matrices and how to establish electrical communication between the immobilized Abs and the electrode surfaces. We also reported the use of Ab-modified quartz crystals for piezoelectric sensing of the dynamics of Ab–Ag interactions [17]. In these systems, the electrodes or quartz crystals were modified with layers of the Abs or Ags of interest in order to detect the complimentary antibodies. The electrochemical detection of the Ag by the Ab-immobilized surface was based on the extent of the changes in current due to the Ab–Ag binding. We then demonstrated the use of polymers together with pulsed amperometric detection (PAD) in a flow-injection analysis (FIA) to obtain sensors having detection limits in the low parts-per-billion (ppb) range. The selectivities of these sensors to other structurally similar compounds, ions, or proteins were less than 3%. Specifically, it was possible to control the Ab–Ag interaction using Ab-immobilized conducting polymers coupled to periodic or transient pulsed voltage waveforms. In this paper, we present an overview of the pulsed electrochemical method, the mechanism of the Ab–Ag interactions at sensor surfaces, as well as their analytical capabilities.

### 1.1. Mechanism of sensor–analyte interactions

The interaction of a biochemical species,  $P$ , with a sensor,  $S$ , can be described by the following equilibrium:



The equilibrium constant  $K$  is given by the equation:

$$K = \frac{[SP]}{[P][S]} = k_f/k_b, \quad (2)$$

where  $[SP]$  is the molar concentration of the bound species, while  $[P]$  and  $[S]$  are the molar concentrations of the analytes in the sample and the binding site, respectively. The overall rates of the forward ( $k_f$ ) and backward ( $k_b$ ) reactions, as well as the mass-transport parameters of the species involved in the transduction mechanism determine the time response of the sensor. The free energy change from the reaction in Eq. (1) is given by

$$\partial G = 0 = \partial G^0 + RT \ln \frac{[SP]}{[P][S]}. \quad (3)$$

It therefore follows from Eq. (3) that, upon a change in the sample concentration, the interaction of the species with a sensor will occur if the variation in the standard free energy ( $\delta G^0$ ) is negative and the ratio of the log of  $[SP]/[P][S]$  is less than unity. From a practical point of view, the reaction will be irreversible when the binding equilibrium constant ( $K$ ) is too high (i.e., for  $K > 10^4$ ). Therefore, the sensor will respond in a nonequilibrium manner. Also, from the sensor application point of view, the high value of the binding constant will produce a highly sensitive device, but the irreversible nature of the interaction is a disadvantage as it makes sensor reusability impossible. Consequently, the use of positive potential should encourage sensor–analyte interactions through a negative  $\delta G^0$ , while a negative applied potential should discourage sensor–analyte interactions through a positive  $\delta G^0$ .

## 2. Assay procedure

### 2.1. Sensor preparation steps

In developing an immunosensor, it is first necessary to immobilize an antibody onto the surface of a transducer using either electrochemical, optical, or piezoelectric methodologies. This can be carried out by covalent attachment, physical adsorption, or

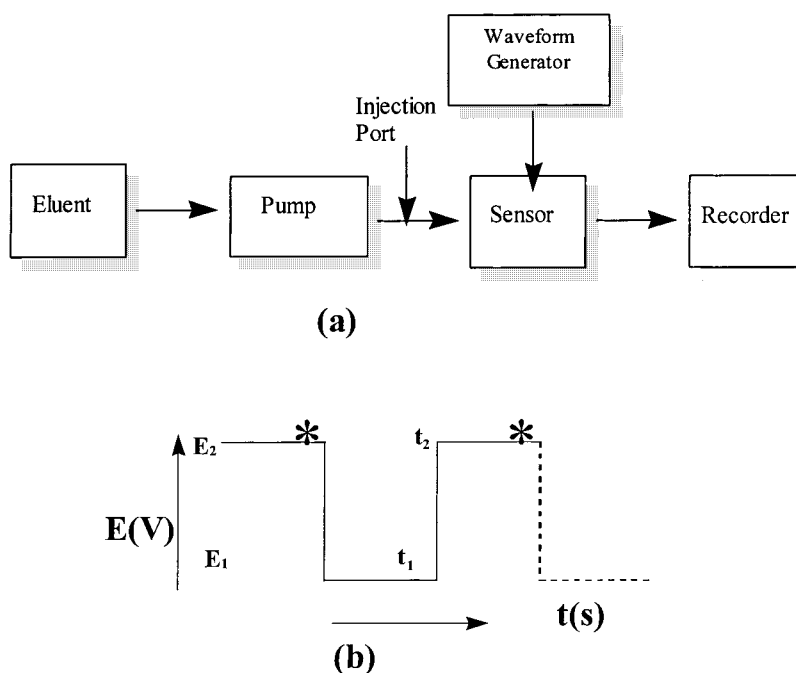


Fig. 1. (a) Experimental setup. (b) The modulation of Ab–Ag reactions using pulsed-potential waveforms.

electrostatic entrapment in a conducting polymer matrix such as polypyrrole (Ppy) [3]. The polymeric support also serves to increase the number of biological molecules at the sensor surface. The use of Ppy is suitable for this purpose because it can be easily prepared on miniaturized components, and its relative high conductivity can be used to generate sensor signals through an applied potential. Finally, it provides relative stability for the immobilized antibody.

## 2.2. Signal generation

After preparation, the sensors can then be used for analyte detection in a static or flow injection mode by applying pulsed potentials to the sensor surface (i.e., between the working electrode and the reference electrode). This involves the movement of the analytes in a stream of eluent into and out of the detection cell. The residence time of the analytes in the detection cell is short (<1 min), and hence the signal generation is fast. A pulsed potential waveform should be employed to modulate this rapid signal as depicted in Fig. 1. In Fig. 1(b),  $E_1$  and  $E_2$  are chosen such that the Ab–Ag interactions are enhanced at  $E_2$  and then reversed at

$E_1$ . The frequency (i.e., pulse width between  $t_1$  and  $t_2$ ) is such that the Ab–Ag interaction does not reach a stage where it becomes irreversible. The electrical signal at that point is obtained by a repetitive sampling of the current at 16.7 ms before the end of the pulse. The current or mass signals obtained are directly related to the rate of the electrochemical reaction occurring at the sensing electrode shown by the following equation:

$$\frac{d[PS]}{dt} = k_f[S]PS_{\max} - (k_f[S] + k_b)PS. \quad (4)$$

This equation is directly related to the analyte concentration in the solution if  $d[PS]/dt$  is plotted against  $P$  and there are no mass transport limitations.

## 3. Results and discussion

### 3.1. Sensor regeneration and analytical performance

Some studies about the regeneration of the sensor surface were carried out using PAD in order to enhance the rate of immunobinding. Fig. 2 shows that with the use of PAD, it is possible to detect real-time

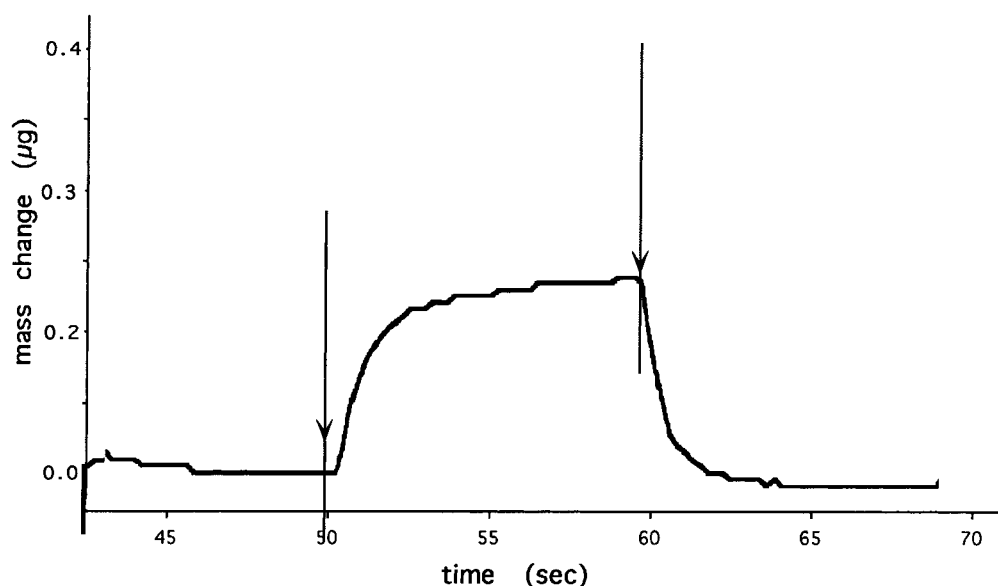


Fig. 2. The reversibility of sensor signals with pulsed potentials at Ppy/Anti-HSA electrodes in the presence of 5 ppm HSA solution in 0.1 M  $\text{NaNO}_3$ . A pulsed-potential waveform of potential ( $E$ ) with time ( $t$ ) employs a variable potential applied between  $E_1$  and  $E_2$ ;  $E_1 = -1.0$  V,  $E_2 = 0.40$  V, pulse width = 60 ms ( $n = 10$ ,  $\text{RSD} = 0.30\%$ , current was sampled 16.7 ms before the end of the pulse).

label-free binding and the release of antibodies at the interface. The result shown was obtained using anti-HSA polymer-modified quartz crystal electrode with a concentration of 5 mg/ml HSA. In these experiments, the analytical signal was generated by applying pulsed-potential waveforms between +0.40 and  $-1.0$  V with a pulse frequency range 120–480 ms. Using these parameters, the oscillating potential reversibly drove the Ab–Ag binding, and the signal returned to the baseline. The selectivity of the system was confirmed for HSA, using bovine serum albumin (BSA), chymotrypsin, ovalbumin, and thaumatin proteins. The injection of these proteins into the Ppy/AHSA system gave responses that were lower in magnitude than those obtained for the corresponding concentrations of HSA. Fig. 3 shows that the sensor may be subject to nonspecific binding, however, this effect is negligible compared to the HSA concentration range.

The practical application of the sensor was demonstrated for the detection of albumin in urine samples. Albumin contents in the range of several tens of mg/l in urine is usually regarded as a sign of abnormality in diabetic patients. In a normal body, the HSA level in urine is about 15–17 mg/l in a 24 h urine collection

[22]. Urine samples were pretreated by first centrifuging at 700 rpm and were later filtered. The samples were made up in the eluent and later injected into the sensor system. Estimation was done by standard addition technique (Fig. 4). The reproducibility of each of the responses was about  $\pm 5\%$  over 10 injections, with an estimated detection limit of about 1 ppm. The present measurement system appears to be useful for real-time detection of urinary HSA at the high concentration possible in an abnormal case. The technique appears to suffer from interference when HSA is present at low levels.

Several techniques for real-time detection have been suggested, including surface plasmon resonance (SPR) [9], quartz crystal microbalances [8], optic and fiber coupling [23] techniques. Each technique has its own merits, but it appears that the use of SPR has generated considerable interest and a commercially available SPR-based system from Pharmacia is now available. In SPR, a thin film of metal–dielectric interface is irradiated with coherent polarized light. The intensities of the oscillating changes induced at the surface, known as nonradiative surface plasmons, decay exponentially in a direction perpendicular to the surface. A plot of reflectivity versus incident angle

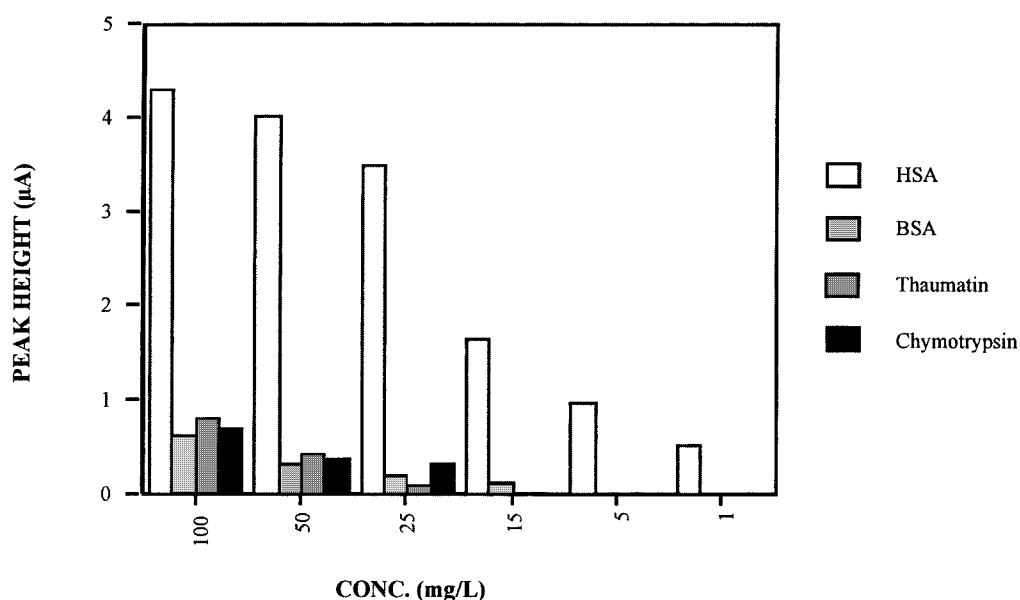


Fig. 3. Selectivity data obtained at Ppy/anti-HSA sensor for the detection of different proteins. Conditions: Ppy/anti-HSA electrode, pulse potentials  $E_1=+0.4$  V,  $E_2=0.00$  V,  $t_1=t_2=120$  ms, flow rate=1 ml/min, injection volume=50  $\mu$ l.

provides the information for the real-time detection of biological reactions. This method is optical in nature and could theoretically meet all of the required attributes of an ideal sensor in terms of its sensitivity and specificity. As in pulsed immunosensors, the specificity could be controlled by the choice of a suitable biological layer since the technique is passive and nondestructive.

However, for SPR to occur, the selective layer must possess a negative component of the optical dielectric permittivity. Many molecular species do not bind very well to the surface of a metal. Moreover, since many

reusable biosensors require acids to remove antigen from antibody, it would be preferable to avoid the use of metals. Other considerations exist such as the sensitivity, the tendency of silver to degrade, and the introduction of diffraction effects especially when using a grating coupling mode [24,25]. Hence there is still a need for a cheap, reusable, and portable biosensing system, and such needs may be addressed with the sensor technology described in this paper.

The performance obtained to date with the sensor utilizing pulsed techniques has been very promising. Table 1 summarizes the analytical performance for

Table 1  
Overview of pulsed amperometric immunosensing systems for selected analytes<sup>a</sup>

Sensor	Immobilization method	LOD	Basis of detection	Remarks	Ref.
HSA	Polymer entrapment	0.5 mg/ml	$\Delta i$	Regenerable	[18]
HSA	Covalent attachment, Pt electrode	$3 \times 10^{-7}$ M	Impedance spectroscopy	Regenerable	[26]
Phenolics	Polymer entrapment	0.5 mg/l	$\Delta I/\Delta m$	Dynamic range three orders of magnitude, reusable	[19]
P-Athau	Polymer entrapment	0.01 mg/ml	$\Delta i$	Regenerable	[20]
PCBs	Polymer entrapment	0.05 ng/ml	$\Delta i$	FIA mode	[3,15]
Atrazine	Covalent attachment	$3 \times 10^{-8}$ M	$\Delta I/\Delta m$	Static mode, regenerable	[21]

<sup>a</sup>LOD=limit of detection,  $\Delta i$ =current changes at Ppy/Ab electrodes, HSA=human serum albumin,  $\Delta m$ =mass changes at piezoelectric crystals,  $\Delta I$ =current changes at inert electrodes, P-Athau=thauatin proteins, PCBs=polychlorinated biphenyls (as Aroclors), FIA=flow injection analysis.

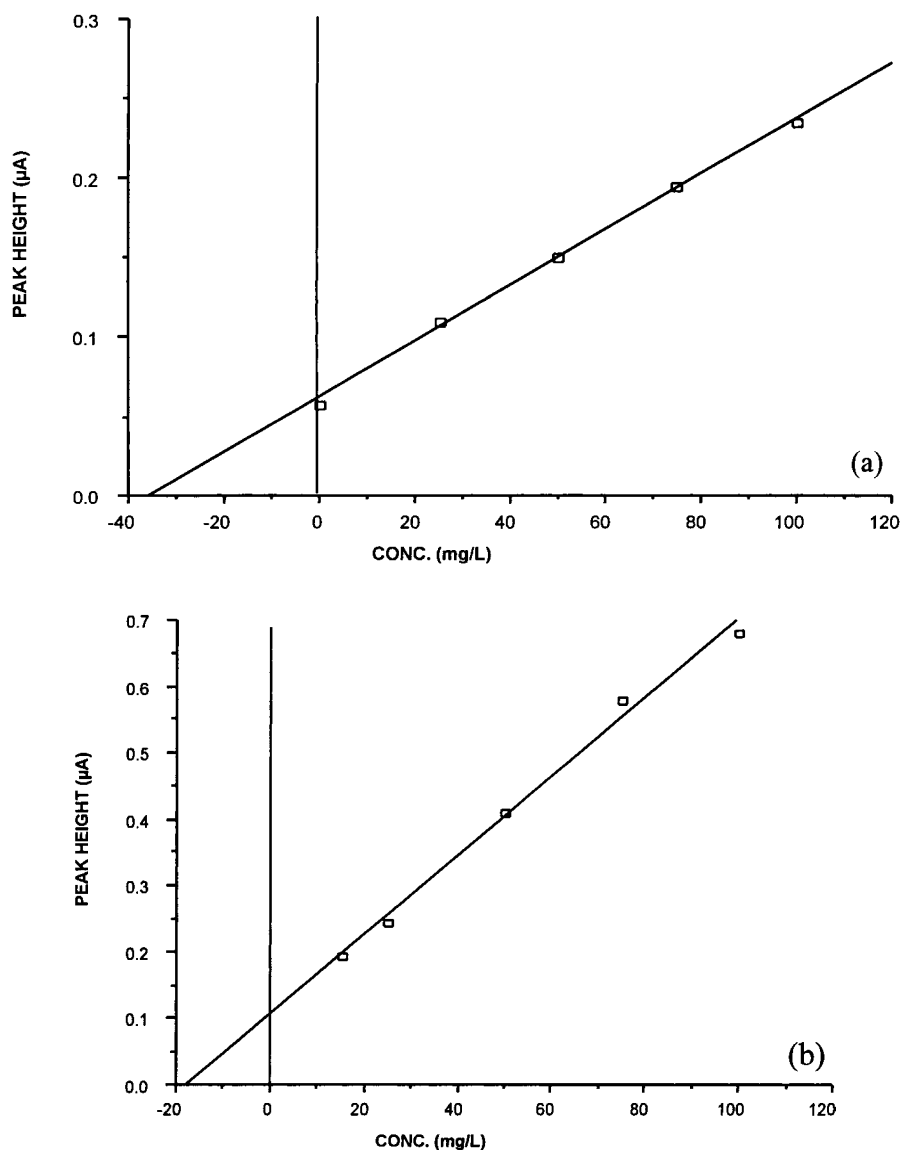


Fig. 4. Typical standard addition curves obtained at Ppy/anti-HSA electrode for albumin determination in urine samples: (a) Sample Y 5461, (b) Sample Y 6358, other conditions as in Fig. 3.

several analytes. The pulsed amperometric immunosensor provides an accurate, reliable method for detecting proteins and small organics in a variety of matrices. Analyte concentration is determined by direct comparison to a standard with a known amount of analytes. The sensor sensitivities can vary with the stability of the selective layer since the activity of the

immobilized antibody is affected by changes in the polymer conductivity. The ability to control the surface conditions of the sensor during the polymerization step will overcome the variations in sensitivities. For environmental applications, the simultaneous analysis of a reference standard as well as unknown samples should address this variability.

#### 4. Summary

The use of PAD provides a rapid, alternative approach to the modulation of antibody–antigen signals at the interfaces. The binding and release of HSA to the modified anti-HSA surface was monitored in real time. The technique has also been demonstrated for a variety of analytical applications, including PCBs, atrazine, phenols, and thaumatin. Reproducible, reversible, and fast response times in the milli-second ranges were obtained.

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